THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 29, Issue of July 18, pp. 18358–18366, 1997
Printed in U.S.A.

Isolation and Molecular Cloning of Wortmannin-sensitive Bovine Type III Phosphatidylinositol 4-Kinases*

(Received for publication, February 12, 1997)

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Agonist-sensitive phosphoinositide pools are maintained by recently-identified wortmannin (WT)-sensitive phosphatidylinositol (PI) 4-kinase(s) (Nakanishi, S., Catt, K. J., and Balla, T. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 5317–5321). Two loosely membrane-associated WT-sensitive type III PI 4-kinases were isolated from bovine adrenal cortex as [^{3}H]WT-labeled 110- and 210-kDa proteins. Based on peptide sequences from the smaller enzyme, a 3.9-kilobase pair (kb) cDNA with an open reading frame encoding a 90-kDa protein was isolated from a bovine brain cDNA library. Expression of this cDNA in COS-7 cells yielded a 110-kDa protein with WT-sensitive PI 4-kinase activity. Northern blot analysis of a human mRNA panel showed a single ~3.8-kb transcript. Peptide sequences obtained from the 210-kDa enzyme corresponded to those of a recently described rat 230-kDa PI 4-kinase. A 6.5-kb cDNA containing an open reading frame of 6129 nucleotides that encoded a 230-kDa protein, was isolated from brain cDNA. Northern blot analysis of human mRNA revealed a major 7.5-kb transcript. The molecular cloning of these novel WT-sensitive type III PI 4-kinases will allow detailed analysis of their signaling and other regulatory functions in mammalian cells.

Phosphatidylinositol (PI)[1] 4-kinases were first recognized as the enzymes that provide PI 4-phosphate for the synthesis of $PI(4,5)P_2$, the precursor of two important intracellular messengers, inositol 1,4,5-trisphosphate and diacylglycerol (1). Early studies indicated that multiple PI kinases are present in cellular homogenates, based on their individual sensitivities to detergents (2, 3). It has been widely accepted that the major PI 4-kinase activity is present in the plasma membrane and regulates the synthesis of phosphoinositides destined for hormone-regulated hydrolysis (3). This tightly membrane-bound activity has been purified from detergent extracts of various membrane sources including the red blood cell membrane (4–7), which contains a ~56-kDa enzyme termed type II PI 4-kinase. Another form of PI 4-kinase, the type III enzyme, was subsequently described in detergent extracts of bovine brain membranes and differed from the type II enzyme by its larger size (over 200 kDa based on sedimentation characteristics), and lower affinity for both ATP and PI (8).

Receptor-mediated regulation of PI 4-kinase activity has been indicated by the rapid increases of PI(4)P levels observed in agonist-stimulated cells (9–11), but few data are available to support direct regulation of this enzymatic activity by either G protein-coupled or growth factor receptors (12–14). Increased PI kinase activity is associated with activated receptor and non-receptor tyrosine kinases and viral oncoproteins (15–17), but this activity phosphorylates PI on the 3- rather than the 4-position of its inositol ring (18). This enzyme, termed type-I PI kinase or PI 3-kinase, contains a 110-kDa catalytic and an 85-kDa regulatory subunit. It produces 3-phosphorylated phosphoinositides by utilizing PI, as well as PI(4)P and $PI(4,5)P_2$, as substrates (see Ref. 19 for a review). Several PI 3-kinases have been purified and cloned (20–24), and some of these, such as the yeast Vps34p, only phosphorylate PI and interact with a 150-kDa subunit. A 210-kDa form of PI 3-kinase has also been reported in *Drosophila* (24).

Recently, the fungal metabolite wortmannin (WT), a potent inhibitor of PI 3-kinases, has been used to define cellular functions that are regulated by PI 3-kinases. Although WT was not believed to inhibit PI 4-kinases (25–27), we observed that micromolar concentrations of the compound abolish the sustained formation of inositol 1,4,5-trisphosphate in agonist-stimulated cells by inhibiting a PI 4-kinase enzyme (28). Further characterization of this soluble (loosely membrane-bound) WT-sensitive PI 4-kinase activity demonstrated its similarity to the type III PI 4-kinase and showed that the bovine brain type III enzyme (as originally described) displays similar WT sensitivity (29). In contrast, the major cellular PI 4-kinase activity, the type II enzyme, was insensitive to WT and thus unlikely to participate in the synthesis of hormone-sensitive phosphoinositide pools (29). The sensitivity of the type III PI 4-kinase(s) to WT (although significantly less than that of PI 3-kinases) raised the possibility that they have structural similarities to PI 3-kinases.

In the present study we report the purification of 110- and 210-kDa WT-sensitive, type III PI 4-kinase enzymes from the bovine adrenal cortex, and the molecular cloning of cDNAs encoding these novel enzymes.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Sepharose, SP-Sepharose (bulk media), and heparin-Sepharose, butyl-Sepharose, MonoQ, and MonoS columns were from Pharmacia Biotech (Uppsala, Sweden). Phosphatidylinositol and ATP were from Fluka (Ronkonkoma, NY) and Sigma, respectively.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U88531 and U88532.

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[1] The abbreviations used are: PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; WT, wortmannin; bp, base pair(s); kb, kilobase pair(s); RACE, rapid amplification of cDNA ends; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid.

[γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham, and [^{3}H]wortmannin-17-ol (19.7 Ci/mmol) was from NEN Life Science Products. Reagents for SDS-PAGE were obtained from Bio-Rad, CA), and restriction enzymes were purchased from Life Technologies, Inc. or New England Biolabs (Beverly, MA). The T/A cloning kit was obtained from Promega (Madison, WI), and the sequencing kits from Amersham. All other chemicals were of HPLC or analytical grade.

Isolation of PI 4-Kinase Activity—In the final purification, 60 bovine adrenal cortices were homogenized in three batches (20 each) in Buffer A (20 mM Tris/HCl, pH 7.3, 1 mM EDTA, 1 mM dithiothreitol, 100 μM AEBSF, and 10 μg/ml leupeptin) containing 1 M NaCl as described previously (29), except that the volumes were upscaled proportionally. All manipulations were performed at 4 °C unless otherwise indicated. After centrifugation (100,000 $\times$ *g*, 90 min), the supernatant was taken to 40% ammonium sulfate (40% saturation) and the precipitated material collected by centrifugation (10,000 $\times$ *g*, 20 min) and the pellets stored at −70 °C until further processing. Ammonium sulfate precipitates were dissolved in 100 ml of Buffer A and dialyzed overnight at 4 °C against 10 volumes of Buffer A containing 30 mM NaCl. The dialyzed protein solution was then diluted to 500 ml with Buffer A/30 mM NaCl and applied to a 5 $\times$ 70-cm DEAE-Sepharose column pre-equilibrated with the same buffer. After loading, the column was washed with 2 liters of Buffer A, 30 mM NaCl and eluted with a linear gradient of 0.03–0.5 M NaCl in Buffer A with a flow rate of 10 ml/min. The PI 4-kinase activity of the fractions was measured as described previously (28). Active fractions were combined and diluted with Buffer B (20 mM MES, pH 6.8, 1 mM EGTA, 0.5 mM dithiothreitol, 100 μM AEBSF, and 1 μg/ml leupeptin) to a conductance of 5 millisiemens/cm. This material was loaded onto an SP-Sepharose column (1.5 $\times$ 50 cm) that was pre-equilibrated with Buffer B. After loading, the column was washed with 300 ml Buffer B and eluted with a linear gradient of 0–1 M KCl in Buffer B at a flow rate of 1 ml/min. Active fractions were combined again and diluted with Buffer A to a conductance of 22 millisiemens/cm and applied to a 5-ml heparin-Sepharose minicolumn, which was equilibrated with 0.2 M NaCl in Buffer A. The column was washed with 20 ml of Buffer A, 0.2 M NaCl and then fitted to an HPLC system (Gilson, Middleton, WI) for elution with a gradient of 0.2–1 M NaCl in Buffer A at a flow rate of 1 ml/min at room temperature. The UV absorption (280 nM) of the eluent was monitored by a Stratos UV-detector (Thomson Instruments, Vienna, VA), and 1-ml fractions were collected on ice. Active fractions were saved and stored at −20 °C with 10% glycerol.

The active fractions from three such purifications were combined and re-chromatographed on the Heparin column to concentrate samples and remove glycerol. Ammonium sulfate was then added to the active fractions (80 mg/ml), and the material was loaded onto a 10-ml butyl-Sepharose minicolumn that was pre-equilibrated with Buffer A/80 mg/ml ammonium sulfate at room temperature. After loading, the column was washed with the same buffer until the absorption of the eluent returned to base line. At this point the column was eluted with Buffer A at 1 ml/min, and 1.5-min fractions were collected. Active fractions were pooled, diluted with three volumes of Buffer A, and loaded onto a Mono Q column (HR 5/5). The column was washed with Buffer A, 50 mM NaCl for 10 min and eluted with a linear gradient of 0.05–0.5 M NaCl in Buffer A over 50 min at a flow rate of 1 ml/min. Two peaks of activity were eluted from this column and were collected and pooled separately. The pooled fractions were diluted with two volumes of Buffer B and loaded onto a Mono S columns (HR 5/5), followed by a 10-min wash with 50 mM KCl in Buffer B and elution with a linear gradient of 0.05–0.5 M KCl in Buffer B over 50 min at 1 ml/min. Fractions that contained the activity were combined, precipitated with 5% (w/v) trichloroacetic acid, and analyzed on an 8% SDS-polyacrylamide gel. After Coomassie staining, the bands that corresponded to the respective enzymes previously identified with [^{3}H]WT binding (see below) were cut out for subsequent peptide sequencing.

Sequencing of Purified Proteins—Coomassie-stained bands at 210 and 110 kDa were excised and subjected to *in situ* proteolytic digestion with modified trypsin (sequencing grade, Promega, Madison, WI) essentially according to the method of Moritz *et al.* (30). Washing steps were performed at 50 °C. The resulting digest was separated at 0.25 ml/min with a gradient described by Fernandez *et al.* (31) on a narrow bore (2.1 $\times$ 250 mm) Vydac 218TP52 column and guard column (Separations Group, Hesperia, CA) at 35 °C using a System Gold HPLC equipped with a model 507 autosampler, model 126 programmable solvent module, and model 168 diode array detector (Beckman, Fullerton, CA). The column effluent was monitored at 215 and 280 nm, and fractions collected at 30-s intervals were stored at −70 °C. Fractions (125 μl) containing tryptic peptides were applied in 30-μl aliquots to a Biobrene (Applied Biosystems, Foster City, CA)-treated glass fiber filter and dried prior to amino acid sequencing on a model 477A pulsed-liquid protein sequencer equipped with a model 120A PTH analyzer (Applied Biosystems) using methods and cycles supplied by the manufacturer. Data were collected and processed by a model 610A data analysis system (Applied Biosystems). Amino acid sequences were searched in the GCG-Swiss Protein Data base (University of Wisconsin Genetics Computer Group).

[^{3}H]Wortmannin Binding Experiments—Aliquots of the fractions eluted from the various columns were incubated for 20 min at room temperature in a total volume of 100 μl of PBS containing 0.4 μCi of [^{3}H]WT-17-ol. This corresponds to 200 nM WT, a concentration that is sufficient to label proteins with lower affinity to WT than PI 3-kinase. In some cases the samples were preincubated with 10 nM unlabeled WT for 10 min prior to labeling to occupy high-affinity binding sites such as those of the PI 3-kinase(s). Proteins were precipitated with trichloroacetic acid (5% final) and subjected to SDS-PAGE. When ethanol precipitation was used instead of trichloroacetic acid (since WT binding has been reported to be acid-labile), there was no difference in the protein labeling. After fixation and Coomassie staining, the gels were impregnated with EN^{3}HANCE (NEN Life Science Products) solution, and after drying were exposed at -70 °C for 1–2 weeks with Hyperfilm (Amersham).

Molecular Cloning of PI 4-Kinases—A size-enriched cDNA library was created in the pSPORT1 plasmid from bovine brain cortex using sucrose-fractionated (>2.5 kb) poly(A)$^+$-selected RNA and the Superscript™ plasmid system for cDNA cloning (Life Technologies, Inc.) according to the manufacturer's instruction. This library contained 1.25 million primary clones and after amplification was stored in aliquots of glycerol stock at −70 °C.

An oligonucleotide primer was designed from the peptide sequence, QLQSIWEQE, obtained from tryptic fragments of the 110-kDa PI 4-kinase, and an antisense primer based on the conserved KDRHNGN sequence that is common to all known PI 4-kinases. PCR amplification using this primer pair (5′-ctgcaRtctatttgggaRcaag-3′ and 5′-attgttaccgtgtgtctgtcctt-3′) yielded a 300-bp product, which was ligated into the PGEM-Easy plasmid (Promega) and subjected to dideoxy sequencing. This DNA fragment, which encoded an amino acid sequence with high homology to PIK1, was random primer-labeled and used to screen the size-enriched bovine brain cDNA library. Homology search of the data base with the 300-bp sequence also revealed high homology to a rat EST (R46930) (Y-162) that was subsequently provided by Dr. Y. Yamada (NIDR, National Institutes of Health) and on sequencing showed extensive homology with PIK1. The 1.3-kb *Eco*RI/*Xho*I insert of this clone was also used for screening the bovine brain library in subsequent experiments. Positive colonies were isolated and the plasmids cut with *Eco*RI/*Not*I restriction enzymes to determine their insert-size. Several clones were isolated and sequenced, the longest of which (c354) contained a 3-kb insert. The missing 5′ end of the mRNA was obtained by 5′-RACE (version 2.0, Life Technologies, Inc.) following the manufacturer's instructions. The full-length clone used for transfection studies was created by long-range PCR amplification (Elongase, Life Technologies, Inc.) from cDNA prepared by reverse transcriptase (Superscript, Life Technologies, Inc.; Rethrotherm, Epicentre Technologies) from mRNA isolated from cultured bovine adrenal glomerulosa cells. The primers used for this amplification were (5′-aggatccgagaaatggcacacctcag-3′ and 5′-cggcaagctctagagttaccacatgatc-3′), and the 3.3-kb product was ligated into the pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA) after digestion with *Xba*I/*Bam*HI.

The isolation of the cDNA encoding the 210-kDa enzyme was initially attempted by colony hybridization of the brain cDNA library with a PCR-amplified fragment of the human PI4Kα, due to the homology indicated by the peptide sequences obtained from the 210-kDa bovine protein. The longest insert isolated was a 3.3-kb product that lacked a poly(A)$^+$ tail due to the use of *Not*I enzyme during creation of the cDNA library and the presence of an internal *Not*I site after the stop codon in this sequence. The 3′-untranslated region was then obtained by 3′-RACE, and the 5′ end of the transcript was amplified with PCR using primers based on the rat sequence (32) that became available during the course of these studies. Completion of the 5′ sequence information was achieved by amplification from the cDNA library with 5′ primers designed on the flanking plasmid (pSPORT1) sequence. Full-length expressable clones were created by long-range PCR amplification from the cDNA of bovine adrenal glomerulosa cells (primers: 5′cgcggatcctgtgca-gagaccggcatgtgtggag-3′ and 5′-cggaattccacacagagaccggctctgattgtc-3′) and ligation of the 6.3-kb product into the pcDNA3.1(+) plasmid after digestion with *Bam*HI and *Eco*RI.

Northern Blot Analysis—Northern blot analysis was performed uti-

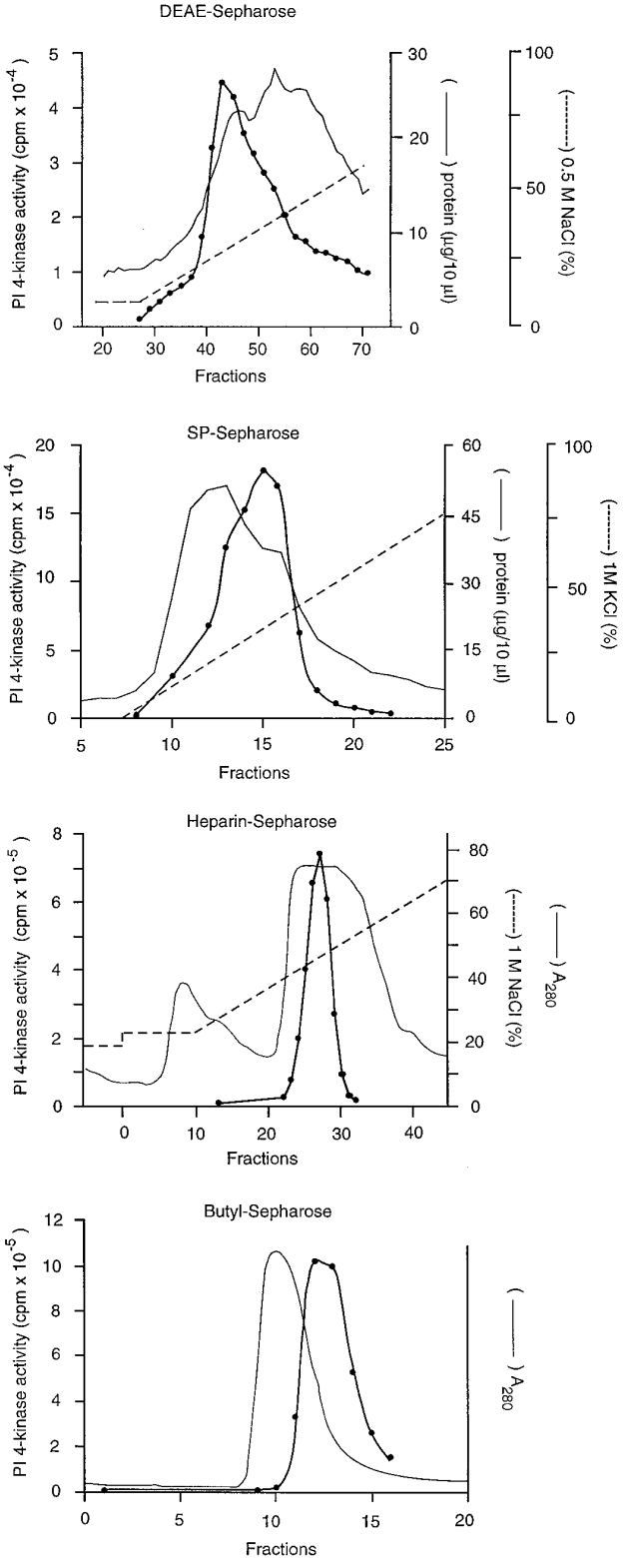

FIG. 1. **Fractionation of WT-sensitive PI 4-kinase activity during sequential chromatographic steps.** Ammonium sulfate precipitates from the soluble fraction of adrenocortical homogenates were dissolved and chromatographed on the indicated ion exchange columns as described under "Experimental Procedures." The PI 4-kinase activities of the effluent fractions were assayed in the presence of Triton X-100 (to inhibit PI 3-kinase) and were found to be inhibited by WT (data not shown).

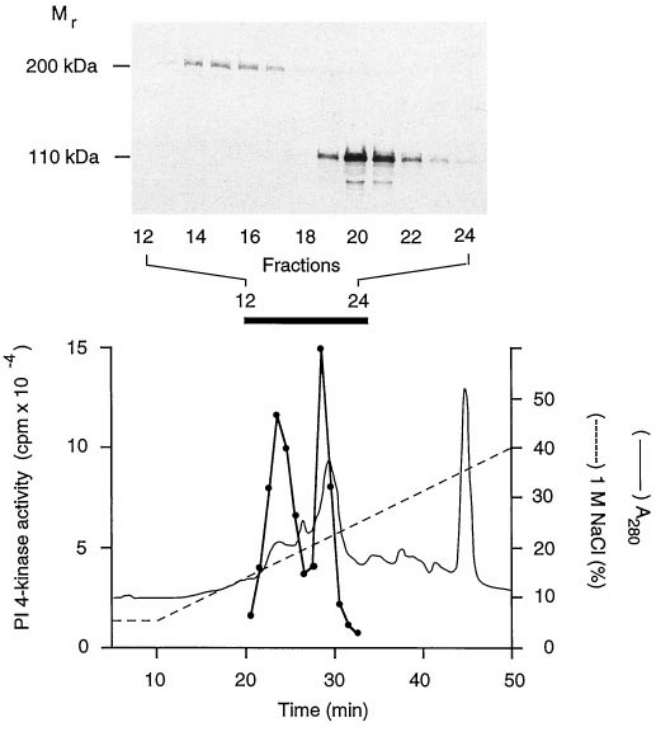

FIG. 2. **Separation of WT-sensitive PI 4-kinase activities by MonoQ ion exchange chromatography.** Enzyme preparations enriched by sequential chromatographies (Fig. 1) were loaded on a MonoQ HR 5/5 column and eluted with a gradient of NaCl as detailed under "Experimental Procedures." PI 4-kinase activity measurements and [^{3}H]WT-binding followed by SDS-PAGE analysis was performed on the effluent fractions. The two peaks of enzyme activity correlated with the labeling of 210-kDa and 110-kDa proteins.

lizing an mRNA panel of several human tissues (CLONTECH, Palo Alto, CA) and the random primer-labeled 1.8-kb fragment of the *Eco*RI digest of one of the clones (c365) (corresponding to nucleotides 1960–3750 of PI4KIIIβ) or the full 2.9-kb insert of a partial clone of PI4KIIIα (c2D5) (3286–6200). After prehybridization at 42 °C in Hybrisol I (Oncor, Gaithersburg, MD), hybridization was performed overnight at 42 °C. The blot was washed several times with increased stringency with a final wash of 0.2 × SSC, 0.1% SDS at 55 °C for 10 min. The blots were analyzed both by autoradiography and by a PhosphorImager (Molecular Dynamics).

Expression of the Enzyme in COS-7 Cells—COS-7 cells were grown to about 70% confluence in Dulbecco's modified Eagle's medium, 10% fetal bovine serum on 10 cm culture dishes. Cells were transfected with 5 ml of Opti-MEM medium containing 10 μg/ml LipofectAMINE (Life Technologies, Inc.) and 5 μg plasmid DNA (pcDNA3.1(+), containing the PCR-amplified clone of PI4KIIIβ). After 8 h the medium was replaced with Dulbecco's modified Eagle's medium/10% fetal bovine serum and culture was continued for selected periods. PI 4-kinase activity was then measured in the soluble fractions after lysing the cells in 500 μl of ice-cold Buffer A containing 150 mM NaCl, followed by sonication and centrifugation at 14,000 × *g* for 30 min at 4 °C. For [^{3}H]WT binding, the supernatants obtained from two such plates were combined, diluted, and applied to 1-ml DEAE-Sepharose columns, and after washing, eluted with 500 mM NaCl in Buffer A. This eluent was then concentrated on Amicon filters and subjected to PI 4-kinase activity measurement and [^{3}H]WT binding followed by SDS-PAGE.

RESULTS

Soluble Extracts of Bovine Adrenal Cortex Contain Two Distinct WT-sensitive PI 4-Kinase—As reported previously (29), two PI 4-kinase activities with native molecular sizes of ~110 and ~200 kDa were identified and partially purified from the bovine adrenal cortex. The catalytic properties of the two components were indistinguishable, raising the possibility that the two peaks represent monomeric and dimeric forms of the same enzyme or that the larger is a heterodimer associated with another protein subunit. Based on the presumed similarity with PI 3-kinases, a purification procedure similar to that used for preparation of PI 3-kinases (33, 34) was employed by se-

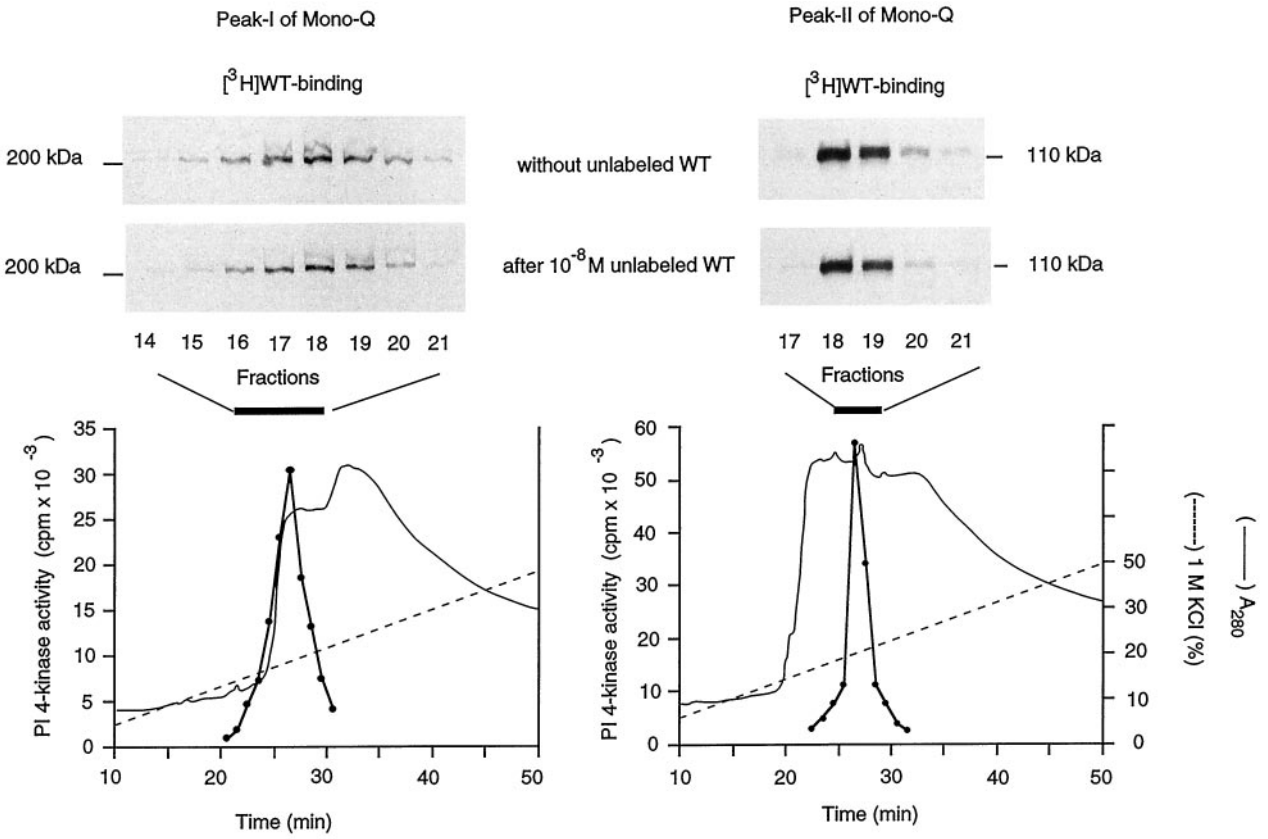

FIG. 3. **Correlation between PI 4-kinase activity and [^{3}H]WT labeling of the effluent fractions of MonoS chromatography of MonoQ-purified 210-kDa (*left*) and 110-kDa (*right*) bovine adrenal PI 4-kinase.** Active fractions eluted from the MonoQ column (Fig. 2) containing the 210-kDa and 110-kDa PI 4-kinase, respectively, were loaded on MonoS HR 5/5 columns and eluted with a KCl gradient as described under "Experimental Procedures." PI 4-kinase activity measurements and [^{3}H]WT-binding followed by SDS-PAGE analysis were performed on the effluent fractions. [^{3}H]WT-binding was only slightly reduced by 10 min preincubation of the samples with 10 nM unlabeled WT (*lower strip* of bands) consistent with the lower affinity of these enzymes for WT. Similar treatment greatly reduced the labeling of PI 3-kinase (data not shown).

quential chromatographies on DEAE-Sepharose, SP-Sepharose, heparin-Sepharose, butyl-Sepharose, MonoQ, and MonoS columns (Fig. 1). Two activities were clearly separated on MonoQ chromatography, and [^{3}H]WT-17-ol-binding (35) was used to correlate the PI 4-kinase activity of the effluent fractions with the [^{3}H]WT labeling of the proteins. As shown in Fig. 2, SDS-PAGE analysis of the WT-labeled proteins revealed that the two peaks of PI 4-kinase activity correlated with two labeled proteins of ~210 and ~110 kDa. The radioactivity bound to these proteins was only slightly reduced by preincubation of the fractions with 10 nM unlabeled WT (Fig. 3). Such treatment greatly reduced the labeling of PI 3-kinase(s) (data not shown), consistent with the higher affinity of these enzymes for WT (29).

Due to their relatively low abundance, these proteins were not purified to homogeneity even after passage through multiple chromatography steps. However, WT labeling allowed their clear identification on SDS gels after Coomassie staining. The two protein bands of interest were then cut out from the gels and digested with trypsin. Only one unequivocal peptide sequence was obtained from the larger enzyme: EFDFFNK, which showed homology to a recently cloned human PI 4-kinase, PI4Kα (36), and to the yeast PI 4-kinase, STT4 (37). Other peptide peaks from the digest were mixtures of peptides that yielded multiple sequences that were homologous to PI4Kα (data not shown). Further purification of the peptides was not attempted because of the minute amounts available. Although the predicted molecular size of PI4Kα is only 97 kDa, we concluded that it probably represents a smaller splice variant of the 210-kDa bovine enzyme, which would be the mammalian homolog of the yeast 200-kDa STT4.

Tryptic digestion of the smaller protein yielded seven unequivocal peptide sequences (LSEQLAHTPTAFK, QLQSIWEQE, VENEDEPVR, LATLPTK, EFIK, EPVFIAAGDIR, and EPGVQA). None of these exhibited homology to any known protein sequence available in the data base. However, a computer alignment of the three human PI 3-kinases (α, β, and γ), and the 125-kDa yeast PI 4-kinase, PIK1, together with one of the sequences, QLQSIWEQE, revealed a possible alignment for the latter (see Fig. 8). This served as the basis for designing primers so that further sequence information could be obtained from this enzyme.

Molecular Cloning of the Two PI 4-Kinases—A primer was designed based on the comparison of the nucleotide sequences encoding QLQSIWEQE, and the corresponding nucleotide sequences of PIK1 and the three PI 3-kinases based on the putative amino acid alignment described above. This primer was used in combination with another primer designed on a conserved sequence (KDRHNGN) that is present in all of the then-known PI 4-kinases (PIK1, STT4, and PI4Kα) but differs slightly in all PI 3-kinases (GDRHNXN). Amplification of a size-enriched bovine brain cDNA library with these primers yielded a 300-bp product with a nucleotide sequence showing extensive homology with PIK1. Screening of the size-enriched bovine brain cDNA library with this product yielded several clones, one of which contained a 3.0-kb insert (c354). Sequencing of this clone confirmed its homology with PIK1, especially within its catalytic domain, and revealed an open reading frame that encoded all seven peptide sequences obtained from the purified protein. Northern blot analysis showed that a

single transcript of ~3.8 kb was present in several tissues (Fig. 6*A*), and 5′-RACE was employed to capture the missing 5′ region of the mRNA. The full-length transcript was found to be 3859 bp with an open reading frame of 2403 nucleotides that encodes a protein of 90 kDa (Fig. 4). Interestingly, one of the isolated clones contained an additional stretch of 48 nucleotides encoding a 16-amino acid serine-rich sequence that is intercalated within one of the peptide sequences obtained from the purified protein, and must represent a splice variant of the enzyme. This sequence was not present in clones that were obtained by PCR for mammalian expression studies and probably represents a minor variant of the enzyme (Fig. 4).

The peptide sequence (EFDFFNK) that was obtained from the larger enzyme indicated its similarity to the 200-kDa yeast PI 4-kinase, STT4 (37) and to a smaller human PI 4-kinase, PI4Kα (36). This and other less certain peptide sequences that were obtained from peptide mixtures of the enzyme digests of the 210-kDa bovine PI 4-kinase suggested that this enzyme is a mammalian homolog of the yeast enzyme, STT4, and a larger form of PI4Kα. Screening of the bovine cDNA library with a cloned fragment (1130–2908) of PI4Kα yielded several clones, one of which contained a 3.3-kb insert. Sequencing of these clones showed their homology with PI4Kα, but revealed a difference in the 5′ end. While this work was in progress, the cloning of a rat 230-kDa PI 4-kinase that showed strong sequence homology with the sequenced bovine clones was reported (32). The 5′ end of the bovine sequence was obtained by PCR, using primers based on the 5′ sequence of the rat enzyme and the longest bovine cDNA clone, and 5′-RACE was used to determine the bovine sequence that corresponded to the 5′ primer (rat) and 5′-flanking sequences. In addition, the 3′-untranslated region of the mRNA was obtained with 3′-RACE, since all the clones were truncated after the stop codon due to a *Not*I site in the sequence and the use of *Not*I during construction of the cDNA library. The full-length sequence of 6520 nucleotides that was reconstructed in this way encoded a 230-kDa protein that showed 92% homology at the protein level with the rat PI 4-kinase enzyme (32) (Fig. 5). Northern blot analysis showed the presence of a prominent ~7.5-kb transcript and minor amounts of smaller transcripts in some tissues, notably the placenta (Fig. 6*B*).

Expression of the 110-kDa PI 4-Kinase in COS-7 Cells—Because of the similarity of the larger bovine PI 4-kinase to the recently characterized 230-kDa rat enzyme, (32), our expression studies were focused on the smaller enzyme. A full-length clone of the 110-kDa enzyme was obtained using RT-PCR of mRNA isolated from bovine adrenal glomerulosa cells with long range PCR and the mammalian expression plasmid pcDNA3.1(+) (see "Experimental Procedures" for details). Plasmid DNA was expressed in COS-7 cells, and kinase activity was measured in the soluble fractions after homogenization and sonication of the transfected cells. As shown in Fig. 7, the transfected cells contained increased PI 4-kinase activity, which after chromatography on DEAE Sepharose minicolumns was completely inhibited by 1 μM WT. Control COS-7 cells that were treated only with LipofectAMINE also contained some endogenous WT-sensitive PI 4-kinase. [^{3}H]WT binding to the extracts obtained from transfected COS-7 cell and subsequent SDS-PAGE showed prominent labeling of a 110-kDa band that was identical to the 110-kDa enzyme purified from bovine adrenal cortex (Fig. 7). Preincubation of the extracts with increasing concentrations of radioinert WT before [^{3}H]WT labeling indicated an affinity that was consistent with the lower sensitivity of this kinase than PI 3-kinase to inhibition by WT (29). Several [^{3}H]WT-labeled bands were present in mock-transfected COS-7 cells and showed no change upon transfec-

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   1 AGCATGATGTCATGTCCTCAGACGAGCAGAGAAATGGCACACCTCAGCAGGGCTGACGAT
  61 GGATGTTTCTGAGCTATTTTTCTTCTCGGTAAAGCCTGGTATGGTGGTTGCTGGCCATTA
 121 TCTTTTGGGTGCGTTGAGTCTAAAAAGGAAGGTAAGAAAAGTATCTATAACTGGAAAAAC
 181 ACATCAAGATTTTAGCACTAAATAAAGGTGAGATGTTCAGGACCTGTGTATGGAGTGTTA
 241 CATGAGGAACAAAATACAGTTTAAAGAGGAAAAACATAAAGGTTACTGGCAAGGCATTTG
 301 AGGGAGATATCGGAGGAGCATGGAGGGAGAAAAAAGCATTTGAAGAGTGGGGACAAGAGA
 361 ATTAAGACCAGAATTGCTTCCCAGTCTATCTGAAGAATTTGTAAACCTATATAAACATAA
 421 CAAAGATAAAGCAACATTTAACAAATACAAGTTAAGAACAGAGGCTAAATTGAGTAACCA
 481 AGCACAGAAGGAGTGAAGAGTAATGTAGGGGTCGGAGGTGGAAGGAGGGAGTCAGGGTAA
 541 GCTTCCTGGGAGGGGATGACTTTTCATCTGAGTTTGAGTGAGTCCCGGAAGACATAAAGA
 601 GAGGGGATGCCTGGGAAGGTGTCCTGGTGAGGGCCAGACCTGAGGTTGGCACCACTAATG
 661 GTGTGTGTGTGTGTCTCCCTTTCTCTTCCTTTCTCCCTCCTTCCTTCTCTAGCTTGGAAG
 721 CTCAAAGTCTGGCTGTGGCCATGGGAGACACGATAGTGGAGCCTGCCCCCCTGAAGCCAA
                      M  G  D  T  I  V  E  P  A  P  L  K  P
 781 CTTCTGAGCCCGCTCCCGGCCCACCAGGGAATAATGGGGGCTCCTTGCTAAGTGTCATCA
     T  S  E  P  A  P  G  P  P  P  G  N  N  G  G  S  L  L  S  V  I
 841 CGGAGGGGGTCGGGGAGCTCTCGGTGATTGACCCTGAGGTGGCCCAGAAGGCCTGCCAGG
     T  E  G  V  G  E  L  S  V  I  D  P  E  V  A  Q  K  A  C  Q
 901 AGGTGCTGGAGAAAGTCAAGCTCTTGCATGGAGGCGTGGCCATCTCTAGCAGAGGCACCC
     E  V  L  E  K  V  K  L  L  H  G  G  V  A  I  S  S  R  G  T
 961 CCCTGGAGCTGGTCAATGGGGATGGTGTGGACAGCGAGATCCGTTGCCTGGACGACCCAC
     P  L  E  L  V  N  G  D  G  V  D  S  E  I  R  C  L  D  D  P
1021 CCGCCCAGATAAGGGAGGAGGAAGATGAGATGGGGGCCACGGTGGCCTCAGGCACAGCCA
     P  A  Q  I  R  E  E  E  D  E  M  G  A  T  V  A  S  G  T  A
1081 AGGGAGCAAGAAGGCGGCGGCAGAACAACTCGGCCAAACAGTCTTGGCTGCTGAGGCTGT
     K  G  A  R  R  R  R  Q  N  N  S  A  K  Q  S  W  L  L  R  L
1141 TTGAGTCGAAACTGTTTGACATCTCCATGGCCATTTCATACCTGTATAACTCCAAGGAGC
     F  E  S  K  L  F  D  I  S  M  A  I  S  Y  L  Y  N  S  K  E
1201 CTGGAGTGCAGGCCTACATTGGCAACCGGCTCTTCTGCTTTCGTAATGAGGATGTGGACT
     P  G  V  Q  A  Y  I  G  N  R  L  F  C  F  R  N  E  D  V  D
1261 TCTATCTGCCCCAGTTGCTTAACATGTACATCCACATGGACGAGGACGTGGGTGATGCCA
     F  Y  L  P  Q  L  L  N  M  Y  I  H  M  D  E  D  V  G  D  A
1321 TCAAGCCCTACATAGTCCACCGCTGCCGCCAGAGCATTAACTTTTCCCTCCAGTGTGCTC
     I  K  P  Y  I  V  H  R  C  R  Q  S  I  N  F  S  L  Q  C  A
1381 TGTTGCTGGGGGCCTACTCTTCAGACATGCACATTTCCACTCAGCGACACTCCCGTGGGA
     L  L  L  G  A  Y  S  S  D  M  H  I  S  T  Q  R  H  S  R  G
1441 CCAAGCTACGGAAGCTGATCCTCTCAGATGAGCTGAAGCCCGCTCACCGAAAGAGGGAGC
     T  K  L  R  K  L  I  L  S  D  E  L  K  P  A  H  R  K  R  E
1501 TGCCCTCCTTGAGCCCAGCCCCCGACACAGGGCTGTCTCCCTCTAAAAGGACCCACCAGC
     L  P  S  L  S  P  A  P  D  T  G  L  S  P  S  K  R  T  H  Q
1561 GCTCTAAGTCAGATGCCACCGCCAGCATAAGTCTCAGCAGCAACTTGAAACGAACAGCCA
     R  S  K  S  D  A  T  A  S  I  S  L  S  S  N  L  K  R  T  A
                GAGCTCTCCTCCAGCACCGAGAGTATTGATAATTCATTCAGTTCC
                 E  L  S  S  S  T  E  S  I  D  N  S  F  S  S
1621 GCAACCCTAAAGTGGAGAATGAGGACGAGCCCGTCAGACTGGCTCCTGAGCGAGAATTCA
     S  N  P  K  V  E  N  E  D  E  P  V  R  L  A  P  E  R  E  F
1681 TCAAGTCCCTGATGGCGATTGGCAAGCGGCTGGCCACACTCCCCACCAAGGAGCAGAAGA
     I  K  S  L  M  A  I  G  K  R  L  A  T  L  P  T  K  E  Q  K
1741 CACAGCGGCTGATCTCAGAGCTCTCCCTGCTCAACCACAAGCTCCCTGCCCGAGTCTGGC
     T  Q  R  L  I  S  E  L  S  L  L  N  H  K  L  P  A  R  V  W
1801 TGCCCACTGCTGGCTTCGACCACCACGTGGTCCGCGTGCCCCACACCCAGGCTGTCGTCC
     L  P  T  A  G  F  D  H  H  V  V  R  V  P  H  T  Q  A  V  V
1861 TCAATTCTAAGGACAAGGCTCCCTACCTGATCTATGTGGAGGTCCTCGAATGTGAAAACT
     L  N  S  K  D  K  A  P  Y  L  I  Y  V  E  V  L  E  C  E  N
1921 TCGACACCACTAGTGTCCCCGCCCGAATCCCCGAGAACCGAATTCGGAGCACCCGGTCTG
     F  D  T  T  S  V  P  A  R  I  P  E  N  R  I  R  S  T  R  S
1981 TAGAGAACCTGCCCGAATGCGGCATCACCCACGAGCAGCGGGCAGGCAGCTTCAGCACCG
     V  E  N  L  P  E  C  G  I  T  H  E  Q  R  A  G  S  F  S  T
2041 TGCCCAACTATGACAACGATGACGAGGCCTGGTCGGTGGATGACATAGGCGAGCTGCAGG
     V  P  N  Y  D  N  D  D  E  A  W  S  V  D  D  I  G  E  L  Q
2101 TGGAGCTCCCTGAAGTGCACACCAACAGCTGTGACAACATCTCCCAGTTCTCCGTGGACA
     V  E  L  P  E  V  H  T  N  S  C  D  N  I  S  Q  F  S  V  D
2161 GCATCACCAGCCAGGAAAGCAAGGAGCCTGTGTTCATCGCAGCAGGTGACATCCGACGGC
     S  I  T  S  Q  E  S  K  E  P  V  F  I  A  A  G  D  I  R  R
2221 GCCTTTCAGAGCAGCTGGCTCACACCCCCACAGCCTTCAAACGAGACCCGGAAGACCCTT
     R  L  S  E  Q  L  A  H  T  P  T  A  F  K  R  D  P  E  D  P
2281 CTGCAGTTGCTCTCAAAGAGCCCTGGCAGGAGAAAGTACGGCGCATTAGAGAGGGTTCCC
     S  A  V  A  L  K  E  P  W  Q  E  K  V  R  R  I  R  E  G  S
2341 CCTATGGCCATCTCCCCAATTGGCGGCTCCTGTCAGTCATCGTCAAGTGTGGGGATGACC
     P  Y  G  H  L  P  N  W  R  L  L  S  V  I  V  K  C  G  D  D
2401 TTCGGCAGGAGCTGTTGGCCTTTCAGGTGTTAAAGCAACTGCAGTCCATTTGGGAACAGG
     L  R  Q  E  L  L  A  F  Q  V  L  K  Q  L  Q  S  I  W  E  Q
2461 AGCGAGTACCCCTGTGGATCAAGCCATATAAGATTCTTGTGATTTCGGCCGACAGTGGCA
     E  R  V  P  L  W  I  K  P  Y  K  I  L  V  I  S  A  D  S  G
2521 TGATTGAACCAGTGGTCAACGCTGTATCCATTCACCAGGTGAAGAAGCAGTCACAGCTCT
     M  I  E  P  V  V  N  A  V  S  I  H  Q  V  K  K  Q  S  Q  L
2581 CCCTGCTCGATTACTTCCTACAGGAGCATGGCAGTTATACCACTGAGGCATTCCTCAGCG
     S  L  L  D  Y  F  L  Q  E  H  G  S  Y  T  T  E  A  F  L  S
2641 CCCAGCGCAAATTTTGTGCAGAGTTGTGCTGGCTACTGCTTGGGCTGCTACCTGCTGCAGG
     A  Q  R  N  F  V  Q  S  C  A  G  Y  C  L  G  C  Y  L  L  Q
2701 TCAAGGACAGACACAATGGGAACATCCTGTTGGACGCAGAAGGCCACATCATCCACATCG
     V  K  D  R  H  N  G  N  I  L  L  D  A  E  G  H  I  I  H  I
2761 ACTTTGGCTTCATCCTATCCAGCTCACCCCGAAACCTGGGCTTTGAGACATCAGCCTTCA
     D  F  G  F  I  L  S  S  S  P  R  N  L  G  F  E  T  S  A  F
2821 AACTGACCACAGAGTTTGTGGACGTGATGGGCGGCCTGGATGGCGACATGTTCAACTATT
     K  L  T  T  E  F  V  D  V  M  G  G  L  D  G  D  M  F  N  Y
2881 ACAAGATGTTGATGCTGCAGGGGCTGATCGCTGCTCGGAAACACATGGATAAGGTGGTGC
     Y  K  M  L  M  L  Q  G  L  I  A  A  R  K  H  M  D  K  V  V
2941 AGATCGTAGAGATCATGCAACAAGGTTCTCAGCTTCCTTGCTTCCATGGCTCCAGCACCA
     Q  I  V  E  I  M  Q  Q  G  S  Q  L  P  C  F  H  G  S  S  T
3001 TCCGCAACCTCAAGGAGAGGTTCCACATGAGCATGACCGAGGAGCAGCTGCAGCTGCTGG
     I  R  N  L  K  E  R  F  H  M  S  M  T  E  E  Q  L  Q  L  L
3061 TGGAGCAGATGGTGGATGGCAGCATGCGGTCTATAACCACCAAACTCTATGACGGCTTCC
     V  E  Q  M  V  D  G  S  M  R  S  I  T  T  K  L  Y  D  G  F
3121 AGTACCTCACCAACGGCATCATGTGACGCCCTCCTCCTCATGCCCCAGCACGATGTGGGG
     Q  Y  L  T  N  G  I  M
3181 GTTCAGGGGACCCTCCCTGGGAAGGCCCTTGGAGAAACCCTAAGCCAGGAAGCCCCACCC
3241 CCCCAGCCATCCACCAAGGGGAATGGAAGGCAAGAAATACAAAGGATCATGTGGTAACCG
3301 TAGAGCTTGCCGGGGCGGGTGGGGAGAGCCAGCTGTGGGGTCCAGACTGACTGGGGCTTC
3361 CTTGCCGCCACCCCAGCTGTGTCAGTATTACCACCTGATGGGCTCCAGGACTCACTGCCC
3421 TCCAGAGAACAGAAGTGATAAGTGTGGGGGGCGCTGGGGCCTCACTTCTCCTCACCAGGG
3481 TCCGAGAGTTTCTTTCCACAGGCCATCCGCTTCATGTATTCTAGGTCCCAGGAAGAAAAG
3541 AGTAGGTTCTTGGTACTGAGGACTTGATCCTGTGGTTGCCTTTGCCATGCTGCTGCCTGT
3601 CCCCTCCCAGGGACTGAGCCCGAGCCCGAGTTCCCCTGGGTAGGTGGGCTTTGGCATAGG
3661 GTTCTGCACTTGCTGAGGTCCCCCATCCCAGAAGTGAAGAGGGAATGATCACGGCCCCTC
3721 CAATCTGAGGGTACTGGCCTAGCCTCGGGGAATTCCTTGCCCTGATCCCTTCCCCACACC
3781 CAGGGAGATAGCTCTCAATTTTTTATTTTTAATTTTTGTTTGAAATAAAGTCCTTAGTTA
3841 GCCATCAAAAAAAAAAAAA
```

FIG. 4. **Nucleotide and predicted amino acid sequence of the 110-kDa type III bovine PI 4-kinase.** The sequence within the *box* shows a minor splice variant of the enzyme as indicated by one of the isolated clones. The peptide sequences obtained from the purified 110-kDa enzyme are *underlined*.

```
1    CCGGCTCCCTGGCTGTGCAGAGACCGGCATGTGTGGAGAAGGTCCAAAAGCTGCTTTGTA
61   TGTGCCCAGTAGATTTCCATGGGATCTTCCAGCTGGATGAGAGGCGGAGAGACGCAGTGA
     M  C  P  V  D  F  H  G  I  F  Q  L  D  E  R  R  R  D  A  V
121  TCGCATTGGGCATCTTTCTCATTGAATCTGACCTACAGCACAAAGACTCTGTGGTTCCTT
     I  A  L  G  I  F  L  I  E  S  D  L  Q  H  K  D  S  V  V  P
181  ACCTTCTTCGACTTCTTAAAGGTCTTCCGAAAGTGTATTGGGTAGAAGAGAGCACAGCTC
     Y  L  L  R  L  L  K  G  L  P  K  V  Y  W  V  E  E  S  T  A
241  GGAAGGGCAGAGGTACCCTCCCGGTGGCCGAGAGCTTGAGCTTCTGCTTGGTCACTCTGC
     R  K  G  R  G  T  L  P  V  A  E  S  L  S  F  C  L  V  T  L
301  TGTCTGACGTGGCTTACAGGGACCCTTCCCTGAGGGGTGAGATCCTAGAGGCGCTGCTGC
     L  S  D  V  A  Y  R  D  P  S  L  R  G  E  I  L  E  A  L  L
361  AGGTCTTGCACGTGCTCCTGGGGATGTGCCAGGCCCTGGAGATTCAAGAGAAAGAGTACC
     Q  V  L  H  V  L  L  G  M  C  Q  A  L  E  I  Q  E  K  E  Y
421  TCTGCAAGCACGCGGTCCCGTGCCTGCTGGGAATCGTGCGCGCCTTTGGGCGGCACAGCA
     L  C  K  H  A  V  P  C  L  L  G  I  V  R  A  F  G  R  H  S
481  GCTCGGAGGACTCGCTCCTGTCCAGGCTGTTCCCCAGAGCTCCTGCGCACTCCCCTCGTG
     S  S  E  D  S  L  L  S  R  L  F  P  R  A  P  A  H  S  P  R
541  TCCCCGAGGAGCTGGAAGGTGTGCGCAGACGGTCCTTCAACGACTTCCGCTCCATCCTCC
     V  P  E  E  L  E  G  V  R  R  R  S  F  N  D  F  R  S  I  L
601  CCAGCAGCCTGCTCACTGTGTGCCAGGAGGGCAGCCTGAAGAGGAAGGCCAGCAGCGCAT
     P  S  S  L  L  T  V  C  Q  E  G  S  L  K  R  K  A  S  S  A
661  CCAGCGTCGCACAGGTCAGCCCTGAGCGTGGGGCACCCCCTCCCAGCCCCCCTGGAGGAT
     S  S  V  A  Q  V  S  P  E  R  G  A  P  P  P  S  P  P  G  G
721  CTGCCTTTCACTGCCTCGAAGCCTCTTACTCACCCAATGGGAGCACCTCGGAATCCGACT
     S  A  F  H  C  L  E  A  S  Y  S  P  N  G  S  T  S  E  S  D
781  ACTACTTCTCTGCCGTCAGCTCCAGCCTCTCCGTCTCTCCTCTCTTCAACGGCGTCACCT
     Y  Y  F  S  A  V  S  S  S  L  S  V  S  P  L  F  N  G  V  T
841  ACAAAGAGTTCAGCATTCCCCTCGAGATGCTCCGGGAACTCTTAAACCTGGTGAAGAAGA
     Y  K  E  F  S  I  P  L  E  M  L  R  E  L  L  N  L  V  K  K
901  TCGTGGAGGAGCCTGTGCTCAAGTCTCTGGACGCAGTGGTCACGGGTGTTATCGAGGCCA
     I  V  E  E  P  V  L  K  S  L  D  A  V  V  T  G  V  I  E  A
961  GCCCCAGTGCCGACCTCTGCCACGGCGCCTTCAGCGACCCCCTGTACGTGGCTGTGCTCA
     S  P  S  A  D  L  C  H  G  A  F  S  D  P  L  Y  V  A  V  L
1021 GGATGCTGCGGGACACCCTGTACTACATGAGAGACCTGCCCACCTCCTTTGTGAAGGAGG
     R  M  L  R  D  T  L  Y  Y  M  R  D  L  P  T  S  F  V  K  E
1081 TGCACGACTTCGTGCTGGAGCAGTTCAACAGCAGCCAGGGGGAGCTCCAGAAGATCCTGC
     V  H  D  F  V  L  E  Q  F  N  S  S  Q  G  E  L  Q  K  I  L
1141 ACGATGCCGACCGGGCCCACAGCGAGCTGAGCCCCCTCAAGCTGCGCTGCCAGGCCAGTG
     H  D  A  D  R  A  H  S  E  L  S  P  L  K  L  R  C  Q  A  S
1201 CCGCCTGCGTGGACCTCATGGTCTGGGCAGTGAAGGACGAGCAGGGTGCAGAGAATCTGT
     A  A  C  V  D  L  M  V  W  A  V  K  D  E  Q  G  A  E  N  L
1261 GCGTCAAGCTATCTGAGAAGCTGCAGTCCAAGACGTCCAGCAAGGTCATCATCGCGCAGG
     C  V  K  L  S  E  K  L  Q  S  K  T  S  S  K  V  I  I  A  Q
1321 TGCCACTGCTCATCTGCTGTCTGCAGGGCCTGGGCCGCCTGTGCGAGCGGTTCCCGGTGG
     V  P  L  L  I  C  C  L  Q  G  L  G  R  L  C  E  R  F  P  V
1381 TGGTGCACTCGGTGACACCCTCCCTGCGGGACTTCCTGGTGGTCCCATCCCCCGTGCTGG
     V  V  H  S  V  T  P  S  L  R  D  F  L  V  V  P  S  P  V  L
1441 TGAAGCTGTACAAGTGCCACAGCCAGTACCACACAGTTGCCGGCAATGACATAAAAATCA
     V  K  L  Y  K  C  H  S  Q  Y  H  T  V  A  G  N  D  I  K  I
1501 GCGTAACCAATGAGCATGCCGAGTCGACCCTAAATGTGGCATCGGGGAAGAAGAGCCAGC
     S  V  T  N  E  H  A  E  S  T  L  N  V  A  S  G  K  K  S  Q
1561 CGTCCATGTATGAGCAGCTTCGGGACATTGCCATCGACAATGTCTGCAGGTGCCTGAAGG
     P  S  M  Y  E  Q  L  R  D  I  A  I  D  N  V  C  R  C  L  K
1621 CGGGCCTGACCGAGGACCCAGTGATCGTGGAGGCCTTCCTGGCCAGCCTCTCCAACCGGC
     A  G  L  T  E  D  P  V  I  V  E  A  F  L  A  S  L  S  N  R
1681 TGTACATCTCCCAGGAGAGCGATAAGGATGCACACCTGATTCCTGACCACACCATCCGAG
     L  Y  I  S  Q  E  S  D  K  D  A  H  L  I  P  D  H  T  I  R
1741 CCTTGGGACACATCGCGGTGGCCCTGAGGGACACCCCAAAGGTCATGGAGCCCATTCTGC
     A  L  G  H  I  A  V  A  L  R  D  T  P  K  V  M  E  P  I  L
1801 AGATCCTGCAGCAGAAATTCTGCCAGCCCCCCTCCCCGCTTGACGTGCTCATCATCGACC
     Q  I  L  Q  Q  K  F  C  Q  P  P  S  P  L  D  V  L  I  I  D
1861 AGCTGGGCTGCCTGGTCATCACCGGAAACCAATATATTTACCAGGAGGTGTGGAACCTCT
     Q  L  G  C  L  V  I  T  G  N  Q  Y  I  Y  Q  E  V  W  N  L
1921 TTCAGCAGATCAGCGTGAAGGCCAGCTCGGTTGTGTACTCGGCCACCAAGGACTACAAGG
     F  Q  Q  I  S  V  K  A  S  S  V  V  Y  S  A  T  K  D  Y  K
1981 ACCATGGCTACAGGCACTGCTCGCTGGCGGTGATCAACGCCCTGGCCAACATCGCGGCCA
     D  H  G  Y  R  H  C  S  L  A  V  I  N  A  L  A  N  I  A  A
2041 ACATCCAGGATGAGCACCTCGTGGACGAGCTGCTCATGAACCTGCTGGAGCTGTTTGTGC
     N  I  Q  D  E  H  L  V  D  E  L  L  M  N  L  L  E  L  F  V
2101 AGCTGGGGCTGGAGGGGAAGCGGGCCAGTGAGCGGGCCAGCGAGAAGGGGCCCGCCCTCA
     Q  L  G  L  E  G  K  R  A  S  E  R  A  S  E  K  G  P  A  L
2161 AGGCCTCCAGCAGTGCGGGGAACCTGGGCGTGCTCATCCCTGTGATCGCAGTGCTCACCC
     K  A  S  S  S  A  G  N  L  G  V  L  I  P  V  I  A  V  L  T
2221 GGCGGCTGCCGCCCATCGAGGAGCCGAAGCCGAGGCTGCAGAAGCTCTTCCGGGACTTCT
     R  R  L  P  P  I  E  E  P  K  P  R  L  Q  K  L  F  R  D  F
2281 GGCTGTACTCGGTGCTGATGGGGTTCGCCGTGGAGGGCTCAGGACTCTGGCCCGAGGAGT
     W  L  Y  S  V  L  M  G  F  A  V  E  G  S  G  L  W  P  E  E
2341 GGTACGAGGGGGTCTGTGAGATCGCCACCAAGTCCCCGCTGCTCACCTTTCCCAGCAAAG
     W  Y  E  G  V  C  E  I  A  T  K  S  P  L  L  T  F  P  S  K
2401 AGCCTCTGCGGTCCGTCCTCCAGTACAACTCGGCCATGAAGAACGACACGGTTACGCCTG
     E  P  L  R  S  V  L  Q  Y  N  S  A  M  K  N  D  T  V  T  P
2461 CCGAGCTGAGTGAGCTGCGCAGCACCATCGTCAACCTTCTGGACCCGGCCCCCGAGGTGT
     A  E  L  S  E  L  R  S  T  I  V  N  L  L  D  P  A  P  E  V
2521 CCGCCCTCATCGGCAAGCTGGACTTCGCCATGTCCACCTACCTGCTGTCCGTCTACCGGC
     S  A  L  I  G  K  L  D  F  A  M  S  T  Y  L  L  S  V  Y  R
2581 TGGAGTACATGAGGGTCCTGCGCTCCAGCGACCCCGCCCGCTTCCAGGTGATGTTGTGCT
     L  E  Y  M  R  V  L  R  S  S  D  P  A  R  F  Q  V  M  L  C
2641 ACTTCGAGGACAAGGCCATCCAGAAGGACAAGTCCGGGATGATGCAGTGTGTGATCGCCG
     Y  F  E  D  K  A  I  Q  K  D  K  S  G  M  M  Q  C  V  I  A
2701 TCGCCGACAAGGTGTTCGACGCCTTCCTGAACATGATGGCGGAGAAAGCCAAGACCAAGG
     V  A  D  K  V  F  D  A  F  L  N  M  M  A  E  K  A  K  T  K
2761 AGAACGAGGAGGAGCTGGAGCGCACGCAGTTCCTGCTGGTCAGCTTCAACCACGTCCACA
     E  N  E  E  E  L  E  R  T  Q  F  L  L  V  S  F  N  H  V  H
2821 AGCGCATCCGCAGGGTCGCCGACAAGTACCTGTCTGGGCTGGTGGACAAGTTCCCCCACT
     K  R  I  R  R  V  A  D  K  Y  L  S  G  L  V  D  K  F  P  H
2881 TGCTCTGGAGCGGGACGGTGCTGAAGACCATGCTGGACATCCTGCAGACCCTGTCCCTGT
     L  L  W  S  G  T  V  L  K  T  M  L  D  I  L  Q  T  L  S  L
2941 CGCTGAGTGCTGACATCCACAAGGACCAGCCATACTACGACATTCCCGACGTGCCCTACC
     S  L  S  A  D  I  H  K  D  Q  P  Y  Y  D  I  P  D  V  P  Y
3001 GCATCACCGTCCCGGACACCTACGAGGCCCGAGAGAGCATCGTCAAAGACTTCGCCGCGC
     R  I  T  V  P  D  T  Y  E  A  R  E  S  I  V  K  D  F  A  A
3061 GCTGCGGGATGATCCTTCAGGAGGCCATGAAGTGGGCGCCCACGGTCACCAAGTCCCACC
     R  C  G  M  I  L  Q  E  A  M  K  W  A  P  T  V  T  K  S  H
3121 TGCAGGAGTACCTGAGCAAGCACCAGAACTGGGTATCGGGGCTGTCGCAGCACACGGGGC
     L  Q  E  Y  L  S  K  H  Q  N  W  V  S  G  L  S  Q  H  T  G
3181 TGGCCATGGCCACCGAGAGCGTCCTGCACTACTCCGGCTACAACAAGCAGAGCACGAGCC
     L  A  M  A  T  E  S  V  L  H  Y  S  G  Y  N  K  Q  S  T  S
3241 TCGGGGCGACACAGCTCACAGAACGGCCAGCCTGTGTCAAGAAGGACTACTCCAACTTCA
     L  G  A  T  Q  L  T  E  R  P  A  C  V  K  K  D  Y  S  N  F
3301 TGGCTTCCCTGAACCTGCGCAACCGCTATGCGGCCGAGGTGTACGGGATGATCCGGTTCT
     M  A  S  L  N  L  R  N  R  Y  A  A  E  V  Y  G  M  I  R  F
3361 CGGACGCCACAGGCCACACATCGGACCTGAACAAGATGATGGTCCAGGAGCTGAAGGCTG
     S  D  A  T  G  H  T  S  D  L  N  K  M  M  V  Q  E  L  K  A
3421 CGCTGGGCGCCGGCGACGCTCAGCAGTACACGCAGGCCATGTTCAAGCTGACGGCCATGC
     A  L  G  A  G  D  A  Q  Q  Y  T  Q  A  M  F  K  L  T  A  M
3481 TCATCAGCAGCAGAGACTGTGACCCGCAGCTCCTCCACCACCTGTGCTGGGGCCCCCTGC
     L  I  S  S  R  D  C  D  P  Q  L  L  H  H  L  C  W  G  P  L
3541 AGATGTTCAACGAGCACGGCATGGAGACCGCCCTGGCCTGCTGGGAGTGGCTGCTGGCCG
     Q  M  F  N  E  H  G  M  E  T  A  L  A  C  W  E  W  L  L  A
3601 GCAAGAACGGGGTGGAAGTGCCGTTCATGCGGGAGATGGCGGGGGCCTGGCAGATGACCG
     G  K  N  G  V  E  V  P  F  M  R  E  M  A  G  A  W  Q  M  T
3661 TGGAGCAGAAGTTCGGCCTGTTTTCTGCGGAGATGAAGGAAGCAGACCCCTTGGCCGCAT
     V  E  Q  K  F  G  L  F  S  A  E  M  K  E  A  D  P  L  A  A
3721 CAGAAGCCAGTCAGCCCAAACCGTGTGCCCCCGAGGTGACCCCTCACTACATCTGGATCG
     S  E  A  S  Q  P  K  P  C  A  P  E  V  T  P  H  Y  I  W  I
3781 ACTTCCTGGTACAGCGGTTTGAGATTGCCAAGTACTGCAGTTCCGACCAGGTGGAGATCT
     D  F  L  V  Q  R  F  E  I  A  K  Y  C  S  S  D  Q  V  E  I
3841 TCTGCAGCCTCCTGCAGCGCTCCCTGTCCCTGAGCATCGGGGGCACCGCGGGCAGCATGA
     F  C  S  L  L  Q  R  S  L  S  L  S  I  G  G  T  A  G  S  M
3901 ACCGGCATGTGGCGGCCATCGGGCCCCGCTTCAAGCTGCTGACCCTCGGCCTGTCCCTGC
     N  R  H  V  A  A  I  G  P  R  F  K  L  L  T  L  G  L  S  L
3961 TGCACGCGGACGTGCTGCCCAACGCCACCATCCGCAACGTGCTCCGCGAGAAGATCTACT
     L  H  A  D  V  L  P  N  A  T  I  R  N  V  L  R  E  K  I  Y
4021 CCACCGCCTTTGACTACTTCAGCTGCCCGCCGAGGTTCCCGACCCAGGGGGAGAAGAGGC
     S  T  A  F  D  Y  F  S  C  P  P  R  F  P  T  Q  G  E  K  R
4081 TGCGTGAGGACATCAGCGTGATGATCAAGTTCTGGACCGCAATGTTCTCAGACAAGAAGT
     L  R  E  D  I  S  V  M  I  K  F  W  T  A  M  F  S  D  K  K
4141 ACCTGACCGCCAGCCAGCTCGTTCCCCCAGACAACCAGGACACCCGGAGCAACCTGGACA
     Y  L  T  A  S  Q  L  V  P  P  D  N  Q  D  T  R  S  N  L  D
4201 TCGCGGTCGGCTCTCGCCAGCAGGCCACGCAGGGCTGGATCAACACGTACCCGCTGTCCA
     I  A  V  G  S  R  Q  Q  A  T  Q  G  W  I  N  T  Y  P  L  S
4261 GCGGCATGTCCACCATCTCCAAGAAATCGGGCATGTCCAAGAAGACCAACCGGGGCTCCC
     S  G  M  S  T  I  S  K  K  S  G  M  S  K  K  T  N  R  G  S
4321 AGCTGCACAAGTATTACATGAAGCGCCGGACGCTGCTGCTGTCACTGCTGGCCACCGAGA
     Q  L  H  K  Y  Y  M  K  R  R  T  L  L  L  S  L  L  A  T  E
4381 TCGAGCGCCTCATCACCTGGTACAACCCGCTGTCCGCCCCGGAGCTGGAGCTGGACCAGG
     I  E  R  L  I  T  W  Y  N  P  L  S  A  P  E  L  E  L  D  Q
4441 CGGGGGAGAGCAGCGTGGCCAACTGGAGGTCCAAGTACATCAGCCTGAGCGAGAAGCAGT
     A  G  E  S  S  V  A  N  W  R  S  K  Y  I  S  L  S  E  K  Q
4501 GGAAGGACAACGTGAACCTCGCCTGGAGCATCTCGCCCCACCTGGCCGTGCAGCTGCCAG
     W  K  D  N  V  N  L  A  W  S  I  S  P  H  L  A  V  Q  L  P
4561 CCAGGTTCAAGAACACGGAGGCTATCGGCAACGAGGTGACACGTCTGGTGCGCTTGGACC
     A  R  F  K  N  T  E  A  I  G  N  E  V  T  R  L  V  R  L  D
4621 CAGGAGCCGTTAGTGACGTCCCGGAAGCCATCAAGTTCCTGGTCACCTGGCACACCATCG
     P  G  A  V  S  D  V  P  E  A  I  K  F  L  V  T  W  H  T  I
4681 ACGCCGACGCCCCCGAGCTCAGCCACGTGCTGTGCTGGGCGCCCGCGGACCCACCCACAG
     D  A  D  A  P  E  L  S  H  V  L  C  W  A  P  A  D  P  P  T
4741 GCCTGTCCTACTTCTCCAGCATGTCCCCGCCGCACCCCCTCACGGCGCAGTACGGGGTCA
     G  L  S  Y  F  S  S  M  S  P  P  H  P  L  T  A  Q  Y  G  V
4801 AGGTCCTGAGGTCCTTTCCGCCGGACGCCATCCTGTTCTACATTCCCCAGATTGTGCAGG
     K  V  L  R  S  F  P  P  D  A  I  L  F  Y  I  P  Q  I  V  Q
4861 CGCTCAGGTATGACAAGATGGGCTACGTGCGGGAGTACATCCTGTGGGCCGCCTCCCAGT
     A  L  R  Y  D  K  M  G  Y  V  R  E  Y  I  L  W  A  A  S  Q
4921 CCCAGCTACTGGCACACCAGTTCATCTGGAACATGAAGACCAACATCTACGTGGACGAAG
     S  Q  L  L  A  H  Q  F  I  W  N  M  K  T  N  I  Y  V  D  E
4981 AAGGCCACCAGAAGGACCCCGACATCGGTGACCTCCTGGAGCAGCTGGTGGAGGAGATCA
     E  G  H  Q  K  D  P  D  I  G  D  L  L  E  Q  L  V  E  E  I
5041 CAGGCTCCCTCTCCGGCCCGGCCAAGGACTTCTACCAGCGGGAGTTCGACTTTTTCAACA
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5101 AGATCACCAACGTGTCGGCCATCATCAAGCCCTACCCCAAAGGCGACGAGAGGAAGAAGG
     K  I  T  N  V  S  A  I  I  K  P  Y  P  K  G  D  E  R  K  K
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     A  C  L  S  A  L  S  E  V  K  V  Q  P  G  C  Y  L  P  S  N
5221 CCGAGGCCATCGTGCTGGACATCGACTACAAGTCTGGGACGCCCATGCAGAGTGCCGCCA
     P  E  A  I  V  L  D  I  D  Y  K  S  G  T  P  M  Q  S  A  A
5281 AGGCCCCGTATCTGGCGAAGTTCAAGGTGAAACGTTGCGGGGTTAGTGAGCTTGAGAAGG
     K  A  P  Y  L  A  K  F  K  V  K  R  C  G  V  S  E  L  E  K
5341 AAGGCCTGCGGTGCCGCTCAGACCCCGAGGAGGAGGGCAGCATGCAGGAGGCCGACGGCC
     E  G  L  R  C  R  S  D  P  E  E  E  G  S  M  Q  E  A  D  G
5401 AGAAGATCTCCTGGCAGGCGGCCATCTTCAAAGTGGGGGACGACTGCCGGCAGGACATGC
     Q  K  I  S  W  Q  A  A  I  F  K  V  G  D  D  C  R  Q  D  M
5461 TGGCCCTGCAGATCATCGACCTCTTCAAAAACATCTTCCAGCTGGTCGGCCTGGACCTCT
     L  A  L  Q  I  I  D  L  F  K  N  I  F  Q  L  V  G  L  D  L
5521 TCGTCTTCCCGTACCGGGTGGTGGCCACCGCCCCCGGGTGCGGCGTCATCGAGTGCATCC
     F  V  F  P  Y  R  V  V  A  T  A  P  G  C  G  V  I  E  C  I
5581 CCGACTGCACCTCCCGGGACCAGCTGGGCCGCCAGACCGACTTTGGCATGTACGACTACT
     P  D  C  T  S  R  D  Q  L  G  R  Q  T  D  F  G  M  Y  D  Y
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     I  R  S  M  A  A  Y  S  L  L  L  F  L  L  Q  I  K  D  R  H
5760 ACGGCAACATCATGCTGGATAAGAAGGGCCACCTCATCCACATCGATTTCGGCTTCATGT
     N  G  N  I  M  L  D  K  K  G  H  L  I  H  I  D  F  G  F  M
5821 TCGAAAGCTCCCCGGGCGGCAACCTCGGCTGGGAGCCGGACATCAAGCTGACGGACGAGA
     F  E  S  S  P  G  G  N  L  G  W  E  P  D  I  K  L  T  D  E
5881 TGGTGATGATCATGGGGGGCAAGATGGAGGCCACGCCCTTCAAGTGGTTCATGGAGATGT
     M  V  M  I  M  G  G  K  M  E  A  T  P  F  K  W  F  M  E  M
5941 GTGTCCGTGGCTACCTGGCTGTGCGGCCCTACATGGACGCTGTGGTCTCTCTGGTCACTC
     C  V  R  G  Y  L  A  V  R  P  Y  M  D  A  V  V  S  L  V  T
6001 TCATGCTGGACACGGGCCTGCCCTGCTTCCGTGGCCAGACAATCAAGCTCCTCAAGCACA
     L  M  L  D  T  G  L  P  C  F  R  G  Q  T  I  K  L  L  K  H
6061 GGTTCAGCCCCAACATGACGGAGCGAGAGGCCGCGAACTTCATCCTGAAGGTCATTCAGA
     R  F  S  P  N  M  T  E  R  E  A  A  N  F  I  L  K  V  I  Q
6121 ACTGCTTCCTCAGCAACAGGAGCCGGACCTACGACATGATCCAGTACTATCAGAACGACA
     N  C  F  L  S  N  R  S  R  T  Y  D  M  I  Q  Y  Y  Q  N  D
6181 TCCCCTACTGAGGGCGGGGCGGCCGCAATCAAGCTCCGCAGGCGAGCAGGCCGCCGCTTC
     I  P  Y
6241 ATCCGAGGACAAATGGGCTGGAGGTGCCCCTGCCAAGCCGGAGACGCACGGTCCCGGGTG
6301 CCGCCCGTGTGGCCAGAAGGACAATCAGAGCCGGTCTCTGTGTGGGCACGGGCTCTTCTG
6361 CACTGGACACATTTCCCCCCCCCCGTCCTGTCTCAGAGGTACAGGGAGCACGTTCTTAGG
6421 CCCAAAAATCAGAACAGTGGTATTTAACCAACGTGAGCAAAATAAAAACAAAACACCGTA
6481 AAAACAAAACACCGCTGTGCTGGAACCTGAAAAAAAAA
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FIG. 5. **Nucleotide and predicted amino acid sequence of the 210-kDa type III bovine PI 4-kinase.** Only the unequivocal peptide sequence obtained from the purified 210-kDa protein is shown *underlined*.

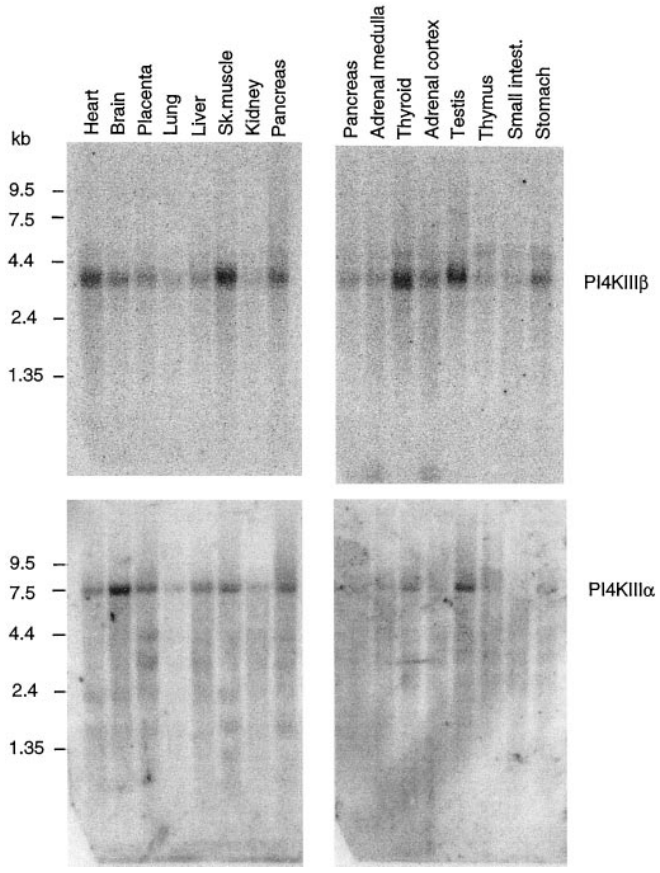

FIG. 6. **Northern hybridization of human mRNA from several tissues with ^{32}P-labeled probes for the 110-kDa (PI4KIII*β*, *upper panels*) and the 210-kDa (PI4KIII*α*, *lower panels*) bovine adrenal PI 4-kinase.** Membranes containing 2 μg of poly(A)$^+$-selected human RNA (CLONTECH) were hybridized with random-prime-labeled cDNA probes for the respective enzymes as described under "Experimental Procedures." After washing (0.2 SSC, 0.1% SDS, 55 °C), the membranes were subjected to analysis by a PhosphorImager after 24 h of exposure.

tion. The most prominent of these was a large protein of >250 kDa that is probably a member of the family of PI kinase-related enzymes (38). The endogenous 210-kDa kinase of COS-7 cells showed only very faint labeling, and the endogenous 110-kDa labeled band is probably a mixture of PI 3- and 4-kinases.

DISCUSSION

Phosphorylation of phosphatidylinositol by PI 4-kinases has long been considered as the initial reaction for the synthesis of membrane phosphoinositides that serve as precursors for the agonist-stimulated formation of inositol 1,4,5-trisphosphate and diacylglycerol. While the most abundant cellular PI 4-kinase, the tightly membrane-bound type II enzyme, was believed to be the most likely candidate to perform this function, our recent studies revealed that the sustained formation of hormone-sensitive inositide pools requires the participation of a WT-sensitive PI 4-kinase enzyme (28). Our analysis of the sensitivity of the various cellular PI 4-kinases to WT also showed that only the type III and not the type II enzyme(s) show such sensitivity (29). Purification of the WT-sensitive, loosely membrane-associated type III PI 4-kinase from the bovine adrenal cortex revealed it to be a mixture of two major activities with molecular sizes of ~200 and 110 kDa (29). [^{3}H]WT labeling and subsequent SDS-PAGE analysis confirmed the existence of two separate proteins of 210 and 110 kDa, both of which showed catalytic properties characteristic of type III PI 4-kinases and similar WT sensitivities (29).

Based on the expected similarity between these enzymes and PI 3-kinases, we employed a purification scheme that was successful for the isolation of PI 3-kinases (33, 34). Although these separation methods were also applicable to the PI 4-kinases, the stronger interaction of these enzymes with the ion exchangers did not allow as efficient separation from the majority of proteins as in the case of PI 3-kinases. Nevertheless, a sufficient amount of protein was obtained to permit petide sequences to be obtained from direct digests of the SDS gel slices containing the two proteins of interest.

Isolation and analysis of cDNA clones encoding these two enzymes confirmed their identity with the purified proteins and revealed that they were mammalian homologs of the yeast STT4 and PIK1 enzymes. Although the overall homology compared with the respective yeast enzymes is low (24% and 16% on the protein level for the bovine 110-kDa enzyme *versus* PIK1 and the 210-kDa enzyme *versus* STT4, respectively), these enzymes show a large degree of conservation within their lipid kinase/protein kinase and lipid kinase unique domains. However, sequence comparison within the kinase domain clearly defined two groups of the type III PI 4-kinases (Fig. 8 and Ref. 36). The marked homology of the 210-kDa enzyme with the much shorter (97 kDa) human PI4Kα enzyme described by Wong and Cantley (36) is consistent with the possibility that the latter might represent a splice variant of the human homolog of the bovine enzyme. The reason for the difference between the catalytic properties of the 210-kDa enzyme (type III) and those reported for the expressed PI4Kα (type II) is not clear at present. However, while these studies were in progress, a novel cDNA that encodes a 230-kDa rat PI 4-kinase was isolated by homology cloning (32) and the purification and partial cloning of a 200-kDa bovine brain PI 4-kinase was reported (39). Although their WT-sensitivities were not examined, both of these larger enzymes were identified as type III PI 4-kinases that are very similar or identical to the 210-kDa enzyme reported in the present study. Based on these findings, we propose the term PI4KIIIα to denote the identity of this larger WT-sensitive enzyme as a type III form with homology to human PI4Kα, and PI4KIIIβ for the 110-kDa bovine PI 4-kinase.

The cDNA sequence encoding the smaller WT-sensitive enzyme, PI4KIIIβ, contained all seven peptide sequences in an open reading frame for a 90-kDa protein. Although this enzyme is a homolog of the yeast PIK1, its sequence similarity is confined largely to the C-terminal third of the molecule that contains the catalytic domain (Fig. 8*A*). Unlike the yeast enzyme, in which the lipid kinase unique domain is located near the N-terminal end, in the bovine enzyme this domain is more distantly positioned from the N terminus (Fig. 8*B*). Interestingly, in all three PI 3-kinases (α, β, and γ), as well as in the large PI 4-kinases, this domain is even further away from the N-terminal part of the molecule (36). PI4KIIIβ also contains a proline-rich sequence in its N-terminal region (Fig. 8). This sequence may promote the interaction of this enzyme with SH3 domains (40), but may also serve as an N-terminal processing signal as suggested in the case of cytochrome P450 enzymes (41). This kinase could also interact with membranes through the putative myristoylation site at its N-terminal end. In contrast, interaction of the larger enzyme, PI4KIIIα, with membranes may also be aided by its putative plekstrin homology domain located between its lipid kinase unique and lipid kinase/protein kinase catalytic domains as described for PI4Kα and STT4 (36). The shorter bovine PI 4-kinase, PI4KIIIβ, showed closest homology to PI3Kγ of the PI 3-kinase family members. We found no sequence homology with PI 3-kinases that would suggest the interaction of this protein with the p85 regulatory protein of PI 3-kinase, and no indication was found

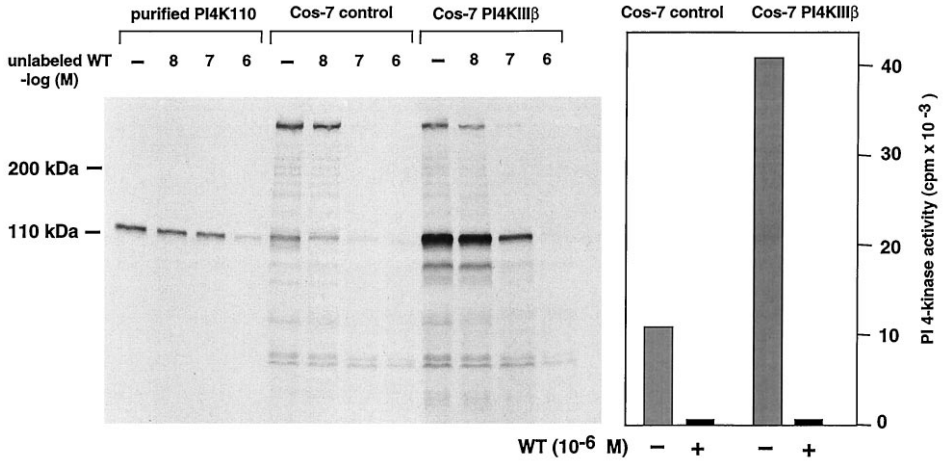

FIG. 7. **Expression of the 110-kDa bovine PI 4-kinase in COS-7 cells.** COS-7 cells were grown to 70% confluence and transfected with LipofectAMINE reagent and the pcDNA3.1(+) plasmid containing the PCR-amplified insert encoding PI4KIIIβ. After 48 h, cells were harvested and sonicated as detailed under "Experimental Procedures." The soluble fractions from control and transfected COS-7 cells were applied to DEAE-Sepharose minicolumns (for separation from WT-insensitive PI 4-kinases) and eluted with 0.5 M NaCl in Buffer A. After concentration, samples were assayed for PI 4-kinase activity in the presence and absence of 1 μM WT (after 10 min of preincubation) or were subjected to [^{3}H]WT-binding after a 10-min preincubation in the presence of increasing concentrations of unlabeled WT. Partially purified 110-kDa PI 4-kinase was also labeled with [^{3}H]WT. Samples were resolved on 10% SDS-polyacrylamide gels and after impregnation with EN^{3}HANCE solution were exposed to x-ray films for 2 weeks at −70 °C.

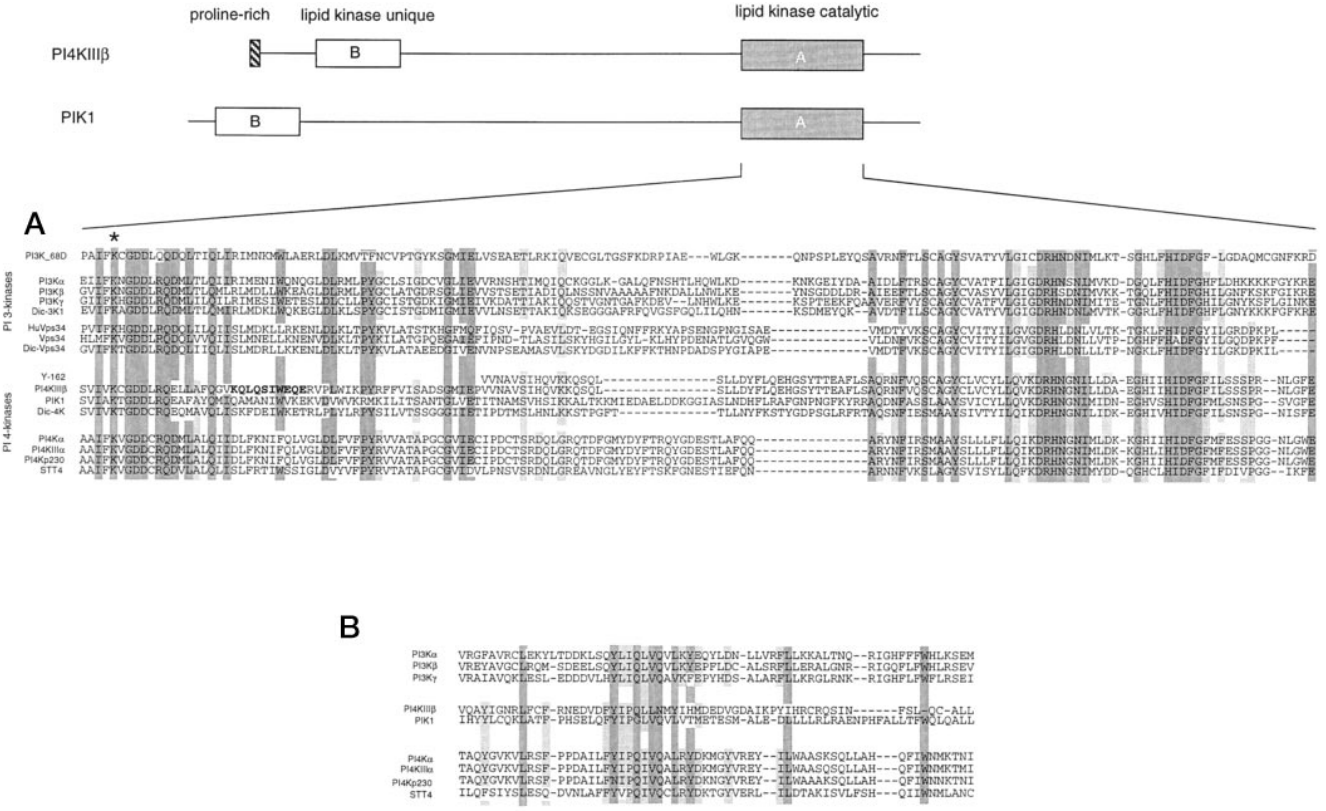

FIG. 8. **Comparison of the amino acid sequences of identified PI 3- and PI 4-kinases within their catalytic lipid kinase/protein kinase domain (*A*) or lipid-kinase unique domain (*B*).** Conserved residues that are present in all or most of these enzymes are indicated by the *dark areas*, and those that are conserved within groups are shown on a *light gray* background. The Lys residue to which WT binds covalently in PI 3-kinases (42) is labeled with an *asterisk*. *PI3Kα, -β*, and -γ as well as *huVps34p* and *PI4Kα* are human sequences (Refs. 20, 36, 21, 49, and 22, respectively), *PI3K_68D* is a sequence from *Drosophila* (24), while *Vps34p*, *PIK1*, and *STT4* are yeast sequences (Refs. 50, 43, and 37, respectively). *Dic-3K1* and *Dic-4K* are the DdPIK1 and DdPIK4 sequences described in *Dictyostelium* (23). *PI4Kp230* is the sequence from the 230-kDa rat PI 4-kinase (32), and *Y-162* is the sequence of the rat EST provided by Dr. Y. Yamada.

during purification for the existence of heterodimers of the PI 4-kinases. The relatively large difference between the calculated molecular size of the protein and its apparent size on SDS-PAGE raises the possibility that the enzyme undergoes posttranslational modification. Similarly, the yeast enzyme PIK1 shows a larger apparent size on SDS gels (125 kDa) than its calculated molecular size (119 kDa).

Both the 110- and 210-kDa enzymes were found to be sensitive to WT, although at higher concentrations than those needed to inhibit PI 3-kinases (29). A recent study identified Lys-802 of PI3Kα as the site to which WT binds covalently within the putative ATP-binding domain (42), probably with

the help of additional interactions with other residues, including Glu-821, Ser-919, and His-936 of PI3Kα (42). Although the residue corresponding to Lys-802 is highly conserved among all PI kinases, including the two enzymes described in this study, WT sensitivities show great variations even within PI 3-kinases. This suggests that the stabilization of WT binding by other residues is an important determinant of the inhibitory potency of this compound. Interestingly, none of the additional residues named above are conserved between PI 3- and PI 4-kinases. It is important to note that the WT sensitivity of these enzymes is dependent on the experimental conditions, in particular on ATP concentration, incubation time, and pH. Since both of the enzymes have a high K_m for ATP, it is quite likely that at the prevailing ATP concentrations in intact cells, their WT sensitivity is even lower than under *in vitro* conditions. These factors must be considered when interpreting data on the WT sensitivity of cellular responses.

The physiological roles and modes of regulation of the multiple PI 4-kinases are not clear at present. However, our data on the WT sensitivity of the maintenance of agonist-sensitive $PI(4,5)P_2$ pools indicate that one or both of the currently described enzymes participates in this process. In the yeast, deletion of PIK1 but not STT4 is lethal. The latter mutant has an osmolarity-dependent phenotype (37, 43) and increased staurosporine sensitivity, indicating its possible connection with PKC-dependent pathways. PIK1 has also been cloned from *Saccharomyces cerevisiae* with the aid of antibodies raised against the nuclear pore complex and is presumably also present in the nucleus (44), but its function (if any) there is not known. The localization of the epitope-tagged 230-kDa rat PI 4-kinase was largely Golgi-associated in overexpressing COS cells (32), but this could reflect the artificially high production of the protein in such cells. The role of the very abundant WT-resistant type II PI 4-kinase(s) is even more enigmatic, as well as its relationship to the WT-sensitive PI 4-kinase enzymes or to the yeast enzymes. The increasing number of enzymes that are known to be regulated by various phosphorylated inositides, mostly via their plekstrin homology domains (45–48), indicates that regulation of inositide synthesis at several subcellular locations might be an important means of controlling the assembly and function of active signaling complexes.

In summary, the present results describe the identification, isolation, and molecular cloning of two WT-sensitive PI 4-kinase enzymes from the bovine brain and adrenal cortex. These enzymes are mammalian homologs of two yeast PI 4-kinases, PIK1 and STT4, that appear to have clearly distinct functions in yeast. The cloning of these enzymes should facilitate the clarification of their role(s) and regulation in mammalian cells, and the understanding of the multiple roles of phosphoinositides in cell regulation.

Acknowledgments—We are grateful to Dr. Larry Mertz (Life Technologies, Inc., Gaithersburg, MD) for helpful advice and to Dr. Y. Yamada (NIDR, National Institutes of Health) for providing the rat EST clone. The skillful technical work of Yue Zhang and the assistance of Dr. Hong Ji are also greatly appreciated.

Addendum—Shortly after the completion of this study, Meyers and Cantley (51) and Nakagawa *et al*. (52) reported the cloning of a WT-sensitive human and rat PI 4-kinase, respectively, (40) that are homologs of the bovine PI4KIIIβ described in this study.

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